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# Morphological and biochemical studies of the milt (Spermatozoa) of the snow-trout fish S*chizothorax richardsonii* (Gray)

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## Abstract

**The present study was undertaken to evaluate the biochemical and morphological nature of the milt (=spermatozoa) of the snow-trout fish *Schizothorax richardsonii.* The study was conducted to find out the possibilities of evolving cryopreservation, artificial propagation for better fishery development. Scanning Electron Microscopy was employed to find out the structural details of the sperm cell. Biometric and mobility studies were also done on the sperm cells to obtain their sizes and movements. The present findings showed that *S. richardsonii* has high protein and lipid content in gonads. The spermatozonic characteristics of these fishes can help in maintaining stocks of their populations in natural aquaculture resources.**

**Keywords:** *SEM*, *Milt*, *Biometric studies*, *Motility*

## Introduction

Garhwal Himalayas in India is home to various fish fauna of which *Schizothorax* spp. holds a very significant position. They are key group in the snowfed water of Himalayan belt. In Garhwal Himalayas, *Schizothorax* has three species namely *Schizothorax richardsonii* (Gray), *Schizothorax plagiostomus* (Heckel) and *Schizothorax sinatus* (Heckel). The family Cyprinidae to which the *Schizothorax richardsonii* belong is the richest and most important family of fish and its members are distributed throughout the world comprising 220 genera and 2420 species (Nelson, 2006). Their spawning seasons depend upon various interceptive factors such as photoperiod, temperature, pH, flood, turbidity *etc* (Sunder, 1986). These fishes are widely distributed along the Himalayan region of India, Pakistan, Bhutan, Bangladesh and Indonesia (Menon,1992). The physical appearance of *S. richardsonii* is highly modified accordingly to the fast flowing waters of Garhwal hills. They are bottom-feeders and are well adapted to live in rocky and stony bed with icy cold super oxygenated and fast flowing waters of the Himalayan region. They are mainly found in upper stretches of Alaknanda and Bhagirathi rivers of this region.

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In the present study, *Schizothorax richardsonii* was chosen for the determination of its spermatozonic characteristics. The study was also significant from the fact that it was closely associated with the development of cryopreservation. Therefore, establishing the spermatozonic characteristics is an urgent prerequisite for launching conservation policies. Moreover the study of sperm morphology provides fair amount of idea about the modifications in fishes and their utilities (Billard and Cosson, 1992). Spermatozoa of fishes are characterized by wide divergences in their structural organization which has become field of interest from taxonomical point of view (Jamieson, 1991). Sperm morphology also reflects the mode of fertilization. The biochemical characteristics of the milt (=spermatozoa) is highly correlated with gonadal maturation and development. The possibilities of sperm preservation and their motility studies can help in their proper preservation by providing them suitable conditions.

## Materials and Method

Experiments were carried out on various samples of *S. richardsonii* at Premature, Mature and Post Mature Stage to evaluate the protein and lipid content along with pH and temperature estimation. The ecomorphological adaptations in the fish sperm cells were studied using Scanning Electron Microscopy.

**Specimen Collection**

The samples were collected from River Alaknanda (Lat 30º 13'North, Long 78º 47'East) at an altitude of 1780-2500 feet during various seasons. The live fishes were transported to the laboratory and were kept in a well-aerated hatchery at 15-22 ºC before analysis to get acclimatized to the existing conditions. After correct identification and taking morphometric data at species level, the specimens were properly cleaned.

**Semen collection**

For semen collection, specimens of *S. richardsonii* at various stages were administered with intraperitoneal hormone Ovaprim (Ovaprim Syndel Laboratories, Vancouver Canada) at the rate of 0.2 ml/kg body weight. Semen samples were collected in ice cooled sterilized test tubes after 5 h of hormone administration. During semen collection, attention was paid to prevent contamination by faecal matter, urine *etc*. The tubes were stored at 4 ºC for further analysis.

**Lipid estimation**

The lipid estimation was done by conventional Bligh and Dyer method (1959) in which the weight of the empty vial was first taken in which the lipid is to be weighed. Then chloroform and methanol in equal volume were added; centrifuged at 3300 rpm at 5 $^{o}$C for 10 minutes; decant and chloroform layer was retained. The layer was then passed through 2.5 cm anhydrous sodium sulfate layer using Whatman filter paper 1. The solvent was then removed using rotary evaporator under vacuum at 40ºC.Now the weight of the extracted lipid plus the weight is taken. The lipid weight is taken according to the formula.

Weight of lipid = (weight of container + extracted lipid) − (weight of empty container)

**Protein estimation**

The protein estimation was done according to Lowry's *et al*. (1951) with modifications from Hartree (1972). This modification makes the assay linear over a larger range than the original assay. Gonad fragments were first homogenized. The homogenate of fresh sample was prepared in 20% trichloroacetic acid. It was then centrifuged at 200 rpm for 10 minutes. The supernatant was then used to estimate soluble sample protein and residues to evaluate insoluble protein. Bovine Serum Albumin (BSA 1mg/ml) was used as standard. The absorbance was determined at 660 nm using the calibration curve.

**Electron Microscopy**

Gonad fragments of *Schizothorax richardsonii* were fixed in 2% glutaraldehyde and 4% paraformaldehyde in 0.1 M Phosphate buffer (pH 7.4) for 7 h at 4 ºC. The material was post-fixed for 2h in 1% osmium teraoxide at 4 ºC. The sample was then critical point dried and electromicrographs were obtained using Leo-435 VP scanning electron microscope at 20 KV.

**Statistical Analysis**

Statistical evaluation for the semen parameters was performed by Duncan's multiple range test (DMRT). A P value of $P<0.05$ was considered as statistically significant.

**Biometric Studies**

Biometric measurements were recorded with the help of ocular and stage micrometer on the slides prepared for morphological studies. All lengths were reported in micrometers.

**Motility determination**

The collected milt of the fishes were evaluated for motility, pH and temperature estimation. Spermatozoa motility assessment was carried out by diluting milt with sterile water (1: 50) at room temperature (31°C) on glass slide, observed immediately under an inverted microscope (200 X) (Zeiss, Germany) with a CCD camera attachment. Estimation of spermatozoa motility was started immediately (approximately 15 s) after dilution and the movement was observed till 2 min. The motility was recorded in a computer by using computer aided motility software (Biovis motility software, Expert Vision Pvt. Ltd, India) and computer assisted sperm analyzer (CASA).The assessment was done at two different temperatures (at 4ºC and at 28 ºC) in order to evaluate the effect of temperature on sperm motility. The percentage of total number of observed (immotile and poorly motile) spermatozoa were counted.

## Results and Discussion

The milt collected from different samples was taken in measuring vials and the biochemical characteristics were analyzed. The pH value was recorded more towards alkaline region in mature stage than the immature and post mature stage. The temperature of the collected milt was also comparatively higher than in immature fry and post mature (spent) fishes. The pH, temperature, protein and lipid content at premature, mature and post mature stage are summarized in Table-1 to 3. The

biometric parameters are shown in Table-4. The spermatozoa of teleost fishes are characterized by wide divergences and structural organization which is also significant from taxonomical point of view (Jamieson, 1991). Generally spermatozoa of externally fertilizing teleost fishes are differentiated in a head, a small mid-piece and a tail region, but no acrosome. Evidences are mounting to suggest that the amount of milt produced by a fish is of vast significance in fertilization process as large amount of spermatozoa in these externally fertilizing fishes gets wasted due to short mobility and hostile external environment. Hence study of their sperm characteristics is necessary for the development of breeding programmes and conservation policies (Routray *et al.*, 2007).

**Table-1: Biochemical study of milt in *Schizothorax richardsonii* (Premature stage). (May 15-20/2009)**

| S. No. | Wt. of fish (gm) | Length of Fish (mm) | No. of Samples | pH | Temp. ($^0$C) | Protein (mg/gm) | Lipid (mg/gm) |
|---|---|---|---|---|---|---|---|
| **1.** | 160 | 350 | 5 | 7.2 | 15 | 91.00±14.5 | 43.8±4.70 |
| **2.** | 165 | 400 | | 7.3 | 16 | 95.80±15.1 | 45.8±3.99 |
| **3.** | 170 | 410 | | 7.2 | 16 | 94.60±13.9 | 44.5±3.68 |
| **4.** | 200 | 300 | | 7.3 | 16 | 98.90±14.8 | 34.0±3.98 |
| **5.** | 166 | 380 | | 7.2 | 16 | 95.90±14.2 | 45.8±3.68 |
| **Mean value** | **172.2** | **368** | **5** | **7.24** | **15.6** | **95.24±2.85** | **42.78±4.12** |

**Data is expressed as mean± SEM**

**Table-2: Biochemical study of milt in *Schizothorax richardsonii* (Mature stage). (September 15-20/2009).**

| S. No. | Wt. of fish (gm) | Length of Fish (mm) | No. of Samples | pH | Temp.($^0$C) | Protein (mg/gm) | Lipid (mg/gm) |
|---|---|---|---|---|---|---|---|
| **1.** | 218 | 210 | 5 | 7.40 | 18.0 | 189.80±12.2 | 91.50±4.70 |
| **2.** | 280 | 250 | | 7.40 | 18.0 | 190.00±14.5 | 91.20±3.99 |
| **3.** | 300 | 280 | | 7.50 | 18.5 | 191.50±13.2 | 90.00±4.24 |
| **4.** | 420 | 350 | | 7.60 | 19.0 | 190.00±15.2 | 88.50±3.88 |
| **5.** | 450 | 410 | | 7.90 | 20.0 | 192.50±16.2 | 90.50±4.20 |
| **Mean value** | **333.6** | **300** | **5** | **7.56** | **18.7** | **190.76±14.26** | **90.34±4.19** |

**Data is expressed as mean± SEM**

The ultrastructure of the spermatozoa reveals that the flagellum is fastened to the sperm cell by a centriolar complex located in an invagination of the nucleus and the flagellum is separated from the mitochondria by a cytoplasmic channel (Koch and Lambert, 1990). Ovaprim is quite useful for the better production of semen from the fishes. The enhanced mobility of spermatozoa at 4 ºC was observed (> 90%) and mobility occurs for 90 to 97 seconds. The motility and viability of sperm is directly related to the metabolism (Lahnsteiner, 1999). The duration of spermatozoa motility in cyprinids is reported to be till 120 seconds (Suzuki, 1959). The mobility of the spermatozoa is an important indicator of the fertility and a necessary parameter to evaluate the sperm value. Besides this, the mobility of fish spermatozoa is an important factor for its ability to enter into the egg. The results also illustrate that the percentage of motile spermatozoa at low temperature in incubated environment is higher than the non-incubated sperms. Since these fishes inhibit cold waters, the spermatozoa demonstrate better motility at lower temperature. There is no prominent acrosome in *Schizothorax richardsonii* which is typical of all teleosts. However an acrosome like structure was observed on the sperm head of *S. richardsonii*, (Figure -1).High magnification shows that the head

of *Schizothorax richardsonii* is ovoid in shape. The head possess a rough surface throughout (Figure-3).The insertion of the flagellum is central into the head of the sperm cell (Figure-2). The lower temperature of the milt was according to the environment in which the fish lives. The pH value of the milt was more towards alkaline region in mature male gonads but near to neutral in immature and spawned gonads of all fishes examined. The biochemical components *viz.* protein, lipid and water content in *Schizothorax richardsonii* was higher during mature stage. The protein content is reported to be higher in milt than any other tissue *i.e.* intestine, muscles and kidneys in some fishes (Geetha *et al*., 1990). Lipids are quantified more in mature *S. richardsonii* specimens.

**Table-3: Biochemical study of milt in *Schizothorax richardsonii* (Post mature stage). (December 15-20/2009).**

| S. No. | Wt. of fish (gm) | Length of Fish (mm) | No. of Samples | pH | Temp. ($^0$C) | Protein (mg/gm) | Lipid (mg/gm) |
|---|---|---|---|---|---|---|---|
| **1.** | 260 | 496 | 5 | 7.8 | 14 | 110.5±14.2 | 53.2±3.42 |
| **2.** | 210 | 310 | | 7.5 | 14 | 109.6±13.5 | 54.0±4.20 |
| **3.** | 206 | 300 | | 7.5 | 14 | 112.0±14.5 | 53.5±3.99 |
| **4.** | 209 | 310 | | 7.5 | 13 | 112.0±12.5 | 54.4±4.20 |
| **5.** | 160 | 250 | | 7.4 | 13 | 115.0±12.2 | 55.0±3.42 |
| **Mean value** | **209** | **333.2** | **5** | **7.54** | **13.6** | **111.82±13.38** | **54.02±3.84** |

**Data is expressed as mean± SEM**

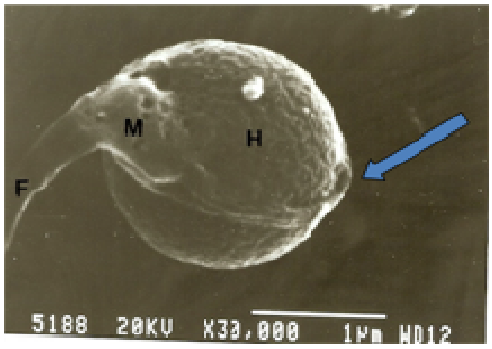


**Figure-1. Sperm head of *Schizothorax richardsonii* showing the ovoid head and acrosome like structure which is unusual of teleosts (x 30, 000). Scale bar= 0.1µm.**

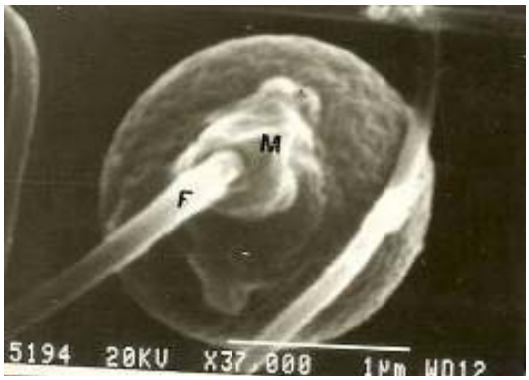


**Figure-2. Shows the central insertion of flagella in the sperm head (x 37, 000). Scale Bar= 1 µm**

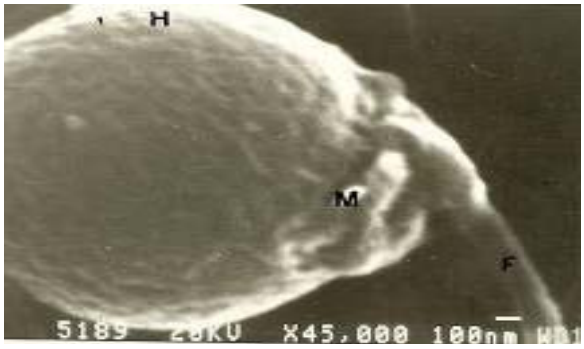


**Figure-3. Shows the rough surface of the sperm cell with no acrosome like structure (x 45,000), Scale bar=100 nm**

This implies the corresponding requirements of more lipids for males as they utilize a lot of their energy in moving around the spawning areas and breeding grounds. The higher length of flagella in these fishes increases the efficiency of undulating movement of sperm in high velocity water current (Stoss, 1983). Scanning Electron Microscopy (SEM) is a powerful tool to describe the morphology of these hill-stream fishes and various modifications which have occurred in them. The fine structure of spermatozoa in fishes is also being studied previously using electron microscopy (Iwamatsu and Ohta, 1981; Lahnsteiner and Patzner 1990; Di Lauro *et al*., 1999; Burns *et al.*, 2002).

However reports on hill-stream cold water fishes is not being done earlier. SEM studies showed the variations between the morphology of these fishes. These divergences in sperm morphology are mainly phylogenetic and do not truly represents the mode of reproduction (Mattei, 1991). Acrosome is completely absent in both the fishes which is typical of all the teleost sperm organization. In the absence of true acrosome, the sperm cells reach the egg plasma membrane through a narrow micropyle. However an acrosome like structutre was observed on the sperm head *of S. richardsonii* which is a

**Table-4: Showing the percentage Mobile and Non-Mobile sperms at two different temperatures**

| S. No. | No. of oozing for each sample | 1 hrs experiment in incubated (1-4 ºC milt 50 times dilution) | | 1 hrs in non-incubated (20-25 ºC) milt with 50 times dilution | |
|---|---|---|---|---|---|
| | | Average percentage of mobile spermatozoa | Average percentage of non mobile spermatozoa | Average percentage of mobile sperms | Average percentage of non-mobile sperms |
| **1** | 6 | 92.5±1.50 | 7.5 | 87.6± 1.2 | 12.4 |
| **2** | 5 | 94.0± 1.75 | 6.0 | 88.5± 1.5 | 11.5 |
| **3** | 9 | 93.0± 1.50 | 7.0 | 90.0±2.0 | 10.0 |
| **4** | 8 | 89.5± 1.50 | 10.5 | 86.5± 1.80 | 13.5 |
| **5** | 7 | 90.5± 1.50 | 9.5 | 85.5± 1.50 | 14.5 |
| **Mean** | **7** | **91.5± 1.55** | **8.1** | **87.62±1.60** | **12.38** |

**Table-5: Biometric parameters of *Schizothorax richardsonii***

| **Sperm** | ***Schizothorax richardsonii*** |
|---|---|
| **HEAD**<br>(i) Length (µm) | Ovoid<br>5.07± 1.49<br>Ellipsoidal |
| **FLAGELLUM**<br>(i) Insertion<br>(ii) Length (µm)<br>(iii) Total Length of spermatozoa(µm)<br>Total No. of Cells counted | <br>Central<br>78.00 ± 3.50<br>83.07 ± 4. 56<br>48 |

**Data expressed as Mean±SEM**

peculiar modification to which the fish has undergone that somewhat differs from other teleosts. A variety of acrosomal structures are known to occur in other fish spermatozoa (Stanley, 1971; Mattei, 1970). The rough surface of *S.richardsonii* may be an ecological adaptation against the fast water current in which the fish dwells. The rough surface helps them to bind to the eggs effectively in the pacedwater currents of the streams and helps the sperm cell to perform the processes of capaciation and egg penetration. The establishment of spermatozoon characteristics can be of immense help to differentiate between the species when morphometric and meristic characters do not resolve its proper species identification. Thus morphologically similar *Schizothorax* spp. (Bahuguna and Bisht, 2005) could be distinguished using spermatozoan characters. These spermato-zonic characterizations of *S.richardsonii* may also help in determining taxonomic ambiguities for future breeding and maintaining stocks of their populations in natural aquaculture resources.

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